

Regulation of *Escherichia coli* Starvation Sigma Factor (σ^S) by ClpXP Protease†

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In *Escherichia coli*, starvation (stationary-phase)-mediated differentiation involves 50 or more genes and is triggered by an increase in cellular σ^S levels. Western immunoblot analysis showed that in mutants lacking the protease ClpP or its cognate ATPase-containing subunit ClpX, σ^S levels of exponential-phase cells increased to those of stationary-phase wild-type cells. Lack of other potential partners of ClpP, i.e., ClpA or ClpB, or of Lon protease had no effect. In ClpXP-proficient cells, the stability of σ^S increased markedly in stationary-phase compared with exponential-phase cells, but in ClpP-deficient cells, σ^S became virtually completely stable in both phases. There was no decrease in ClpXP levels in stationary-phase wild-type cells. Thus, σ^S probably becomes more resistant to this protease in stationary phase. The reported σ^S -stabilizing effect of the *hns* mutation also was not due to decreased protease levels. Studies with translational fusions containing different lengths of σ^S coding region suggest that amino acid residues 173 to 188 of this sigma factor may directly or indirectly serve as at least part of the target for ClpXP protease.

Starvation is a common experience for bacteria in nature (26), and in certain bacteria this stress triggers an elaborate differentiation response, culminating in the formation of spores that are highly resistant, morphologically distinct structures (3, 12, 16). In most other bacteria, this differentiation is more subtle and is not accompanied by a comparable morphological change. Yet 50 or more genes, constituting several temporal classes, have been implicated in starvation-induced development of a resistant cellular state in *Escherichia coli* (19, 25). *E. coli* has been most intensively studied in this respect, but as similar findings have been made in marine vibrios (18), *Salmonella typhimurium* (32), and *Pseudomonas putida* (8, 17), development of a generalized resistant state is probably a universal response of bacteria to starvation.

In *E. coli*, an increase in the concentration of a secondary sigma factor, σ^S (product of the *rpoS* gene), in stationary (starvation) phase appears to be a major trigger for such differentiation. This increase involves regulation at the transcriptional, translational, and post-translational levels (20, 23, 28, 39). With respect to the last-mentioned mechanism, it was recently shown that the stability of σ^S increased markedly in stationary phase (20). In this study, we have investigated the basis of this altered stability. We report that σ^S becomes more stable in exponential phase in the absence of the ClpXP protease from the cells but not that of ClpAP, ClpB, or Lon protease; that the increased resistance of σ^S in stationary phase is not due to a decreased concentration of the ClpXP protease in this phase;

and that a stretch of amino acids near the middle of σ^S appears to be required for its sensitivity to ClpXP activity.

(A report of these findings was presented previously [21].)

MATERIALS AND METHODS

Bacterial strains and construction of *lacZ* translational fusions. The *E. coli* strains and plasmids used in this study are listed in Table 1. Single-copy fusions were prepared as previously described (24, 28, 36). Different-length chromosomal DNA fragments, flanked by appropriate restriction sites to permit directional cloning, were generated by PCR. These fragments contained all the promoters of the *rpoS* gene (19a, 39) plus different lengths of the *rpoS* coding region (28). The fragments were cloned upstream of the *lacZ* gene in the translational fusion vector pRS414 and transferred to the *E. coli* wild-type strain AMS6 (Table 1) chromosome by using phage λ RS45. The resulting strains, AMS302, -303, and -304, contained 314, 563, and 839 nucleotides (nt) of the N-terminal *rpoS* coding region, respectively. Strains AMS154, -156, and -158, constructed previously in this laboratory (28), contained 248, 479, and 750 nt of *rpoS* coding region, respectively (Table 1).

The primers used for constructing AMS302 through AMS304 were as follows. The forward primer used for all the fusions was 5' GCGGAATTCGACGGTC AGCGTATCGTG 3' (–1297 to –1279). The reverse primers were AMS302 (5' GGGGGATCCATTTTACCACCAGACGC 3', +297 to +314); AMS303 (5' GGGGGATCCCTTATGGGACAACCTACGTC 3', +546 to +563); and AMS304 (5' GGGGGATCCAAACCGAATCGACGTGCC 3', +822 to +839). (The underlined sequences for forward and reverse primers denote *EcoRI* and *BamHI* restriction sites, respectively.) The primers used in constructing the (single-copy) transcriptional *clpP-lacZ* fusion strain AMS1109 were 5' GGGCCCGGGTTGC ATGGAACCGTGC 3' (–69 to –53; forward) and 5' GGGGGATCCTCGAA TCGACCAGACC 3' (+687 to +703; reverse) (the underlined sequences represent *SmaI* and *BamHI* sites, respectively).

Strains AMS304Δ1 and AMS304Δ2 are single-copy translational fusions which contained the same chromosomal DNA fragment like AMS304 except for short deletions within the *rpoS* coding region. To construct AMS304Δ1, a PCR product corresponding to nt –1297 to +480 of the *rpoS* gene was made by using the primers 5' GCGGAATTCGACGGTCAGCGTATCGTG 3' and 5' GGGGAGCTCGTTCATAATCGCCCGTTCAATCG 3' (underlined sequences denote *EcoRI* and *SacI* restriction sites, respectively). The fragment was cut with *EcoRI* and *SacI* and inserted into *EcoRI*- and *SacI*-digested pUC19. An additional PCR product corresponding to nt +567 to +839 of *rpoS* was prepared by using primers 5' GGGGAGCTCCTGGACCATGAACCAAGTGCG 3' and 5' GGG GGGATCCAAACCGAATCGACGTGCC 3' (underlined sequences are *SacI* and *BamHI* sites, respectively). This was digested with *BamHI* and *SacI* and ligated to *BamHI*- and *SacI*-digested pUC19 containing nt –1279 to +480 of *rpoS*, generating a deletion from nt +481 to +566. To construct AMS304Δ2, another PCR product corresponding to nt –1297 to +516 of the *rpoS* coding region was generated. The primers used were 5' GCGGAATTCGACGGTCAGCGTATC GTG 3' and 5' GGGGAGCTCTACGATGTGAATCGGCAAAACG 3' (*EcoRI* and *SacI* restriction sites, respectively). This product was inserted into pUC19

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TABLE 1. Strains, phages, and plasmids used in this study

Strain	Characteristics or genotype	Reference
<i>E. coli</i> strains		
AMS6	K-12 (λ^- F $^-$ Δ <i>lac</i>)	35
AMS6A	AMS6 but <i>clpA</i> ; Tet r	This study
AMS6B	AMS6 but <i>clpB</i> ; Km r	This study
AMS6P	AMS6 but <i>clpP</i> ; Cam r	This study
AMS6X	AMS6 but <i>clpX</i> ; Km r	This study
AMS6L	AMS6 but <i>lon</i> ; Tet r	This study
AMS6H	AMS6 but <i>hns</i> ; Km r	This study
AMS6PH	AMS6 but <i>hns/clpP</i> ; Km/Cam r	This study
AMS6C	AMS6 but <i>cbpA</i> ; Km r	This study
AMS6J	AMS6 but <i>dnaJ</i> ; Tet r	This study
AMS6E	AMS6 but <i>grpE</i> ; Tet r	This study
AMS6EL	AMS6 but <i>groEL</i> ; Tet r	This study
AMS154	AMS6 but λ MPN2; Km r	28
AMS156	AMS6 but λ MPN4; Km r	28
AMS158	AMS6 but λ MPN6; Km r	28
AMS302	AMS6 but λ KL102	This study
AMS303	AMS6 but λ KL103	This study
AMS304	AMS6 but λ KL104	This study
AMS154P	AMS154 but <i>clpP</i> ; Cam r	This study
AMS156P	AMS156 but <i>clpP</i> ; Cam r	This study
AMS158P	AMS158 but <i>clpP</i> ; Cam r	This study
AMS302P	AMS302 but <i>clpP</i> ; Cam r	This study
AMS303P	AMS303 but <i>clpP</i> ; Cam r	This study
AMS304P	AMS304 but <i>clpP</i> ; Cam r	This study
AMS304S	AMS304 but <i>rpoS</i> ; Tet r	This study
AMS159	AMS6 but <i>katE::lacZ</i>	28
AMS159P	AMS159 but <i>clpP</i> ; Cam r	This study
AMS60	AMS6 but <i>pexA::lacZ</i>	15
AMS60P	AMS60 but <i>clpP</i> ; Cam r	This study
HYD205	MC4100 but <i>osmY::lacZ</i>	46
HYD205P	HYD205 but <i>clpP</i> ; Cam r	This study
AMS304 Δ 1	AMS6 but λ KL104 Δ 1	This study
AMS304 Δ 2	AMS6 but λ KL104 Δ 2	This study
AMS1109	AMS6 but λ TS1109	This study
AMS1109AT	AMS1109 but <i>relA/spoT</i> ; Km/Cam r	This study
Phages		
λ RS45		36
λ MPN2	λ RS45 with <i>lacZ</i> protein fusion to +248 of <i>rpoS</i>	28
λ MPN4	λ RS45 with <i>lacZ</i> protein fusion to +479 of <i>rpoS</i>	28
λ MPN6	λ RS45 with <i>lacZ</i> protein fusion to +750 of <i>rpoS</i>	28
λ KL102	λ RS45 with <i>lacZ</i> protein fusion to +314 of <i>rpoS</i>	This study
λ KL103	λ RS45 with <i>lacZ</i> protein fusion to +563 of <i>rpoS</i>	This study
λ KL104	λ RS45 with <i>lacZ</i> protein fusion to +839 of <i>rpoS</i>	This study
λ KL104 Δ 1	λ RS45 with <i>lacZ</i> protein fusion to +839 of <i>rpoS</i> but deletion between +481 and +566	This study
λ KL104 Δ 2	λ RS45 with <i>lacZ</i> protein fusion to +839 of <i>rpoS</i> but deletion between +517 and +566	This study
λ TS1109	λ RS45 with <i>lacZ</i> operon fusion to <i>clpP</i> (from -69 to +701)	This study
P1vir		
Plasmids		
pRS414		36
pMMkatF3		29
pGEM-3Z		Promega

and ligated to the PCR product (nt +567 to +839) as already described, resulting in an internal deletion of nt +517 to +566 of the *rpoS* coding region. Control sequencing of selected PCR products showed that no mutations were introduced by the PCR procedure.

Cloning procedures were performed as described by Sambrook et al. (34). P1 vir was used to transduce mutations from one background to another.

Growth conditions and β -galactosidase assay. Cells were grown in glucose (0.05%) M9 medium at 37°C at 200 rpm (33), using exponential-phase cells (grown in homologous medium) as the inoculum. In these media, stationary

phase results from the exhaustion of glucose (35). When specified, this medium was supplemented with antibiotics at standard concentrations (34). Cells were sampled at appropriate intervals for A_{660} and β -galactosidase activity measurements. β -Galactosidase activity was measured as described before (28); the activity is expressed in Miller units.

Immunoblotting. For Western blotting, cells were suspended in sodium dodecyl sulfate (SDS) sample buffer (34) to give a total protein concentration of 35 μ g, as determined by the D_c protein assay (Bio-Rad, Hercules, Calif.). Samples were boiled for 4 min, and their proteins were separated on 10% polyacrylam-

TABLE 2. Western blot quantification of σ^s in exponential-phase wild-type *E. coli* cells (AMS6) and its Lon protease or *clp* mutant derivatives

Mutation	σ^s amt ^a	% of wild-type level
None (wild type)	481	
<i>lon</i>	457	95
<i>clpP</i>	1,639	340
<i>clpA</i>	394	80
<i>clpB</i>	439	90
<i>clpX</i>	1,772	370

^a Amount of σ^s is expressed in arbitrary densitometric units. Each result is the average of three determinations; variation was within 10%.

ide-SDS gels. The proteins were electroblotted onto nitrocellulose membrane filters (Schleicher & Schuell, Keene, N.H.), and the filters were blocked for 2 h with 2% nonfat dry milk. The filters were washed and probed with monoclonal anti- σ^s , polyclonal ClpP, or ClpX polyclonal antibody (7, 10, 27, 31) for 2 h; after being washed, they were incubated with goat anti-mouse or anti-rabbit horseradish peroxidase-conjugated secondary antibody for 1 h. After another wash, the filters were developed with the ECL reagent system according to the manufacturer's protocol (Amersham, Arlington Heights, Ill.). The signal intensity was converted to relative protein levels by densitometry (Image Quant 3.0); control experiments established that a linear relationship existed between concentrations of various proteins and the densitometric readings in these experiments.

Immunoprecipitation. To determine the half-life of σ^s in exponential phase, cells were grown in M9 medium containing 0.3% glucose. After the culture reached an A_{660} of 0.5, 5-ml samples were labeled with 100 μ Ci of L-[³⁵S]methionine (specific activity, 1,000 Ci/mmol) (1-min labeling time for AMS6, 5-min labeling time for AMS6P) (Table 1), followed by a chase with 0.2 mM nonradioactive methionine for 20 s. To determine the half-life in stationary phase, 0.05% glucose M9-grown cells were labeled with L-[³⁵S]methionine 2 h after the onset of the stationary phase (3-min labeling time for AMS6, 5-min time for AMS6P). In both cases, aliquots (0.5 ml) were sampled at appropriate intervals (see Fig. 2) and precipitated with trichloroacetic acid (TCA) (10%) for 30 min.

As a control, AMS304S (*rpoS*) cells, which synthesize a LacZ fusion protein containing 279 amino acids of σ^s , were used. This strain was treated as described above for stationary-phase AMS6. After the nonradioactive methionine chase, the control cells were immediately treated with 10% TCA, as described above. Aliquots (500 μ l) of the mixture were added as an internal standard to each sample. The samples were centrifuged, and the pellets were dissolved in SDS buffer (1% SDS, 50 mM Tris-HCl [pH 8.1], 1 mM EDTA). The immunoprecipitation was done with polyclonal anti- σ^s antibody according to Itoh et al. (13). Densitometry was done as described above. The σ^s levels were corrected with reference to the control band in individual lanes.

RESULTS

Effect of mutation in protease-related genes on σ^s levels. To explore the basis for σ^s lability in exponential-phase *E. coli* cells, we tested the effect of individual mutations in genes connected with two proteolytic activities of *E. coli*, Lon and ClpP (5, 9, 10, 11), on σ^s levels in this phase. The ClpP system requires for proteolytic activity an ATPase subunit, viz., ClpA, ClpX, or possibly also ClpB (37).

lon or the individual *clp* mutations were transduced into the wild-type strain AMS6, and σ^s levels were measured by Western blotting with monoclonal anti- σ^s antibody (31); polyclonal antibody gave similar results. Introduction of the *lon* mutation had no effect (Table 2), but the *clpP* mutation increased exponential-phase σ^s levels fourfold compared with the wild type. Strains containing the *clpA* or *clpB* mutation did not show any change in σ^s levels, but a *clpX* mutation caused the same degree of increase (3.7-fold) as the *clpP* mutation (Table 2).

σ^s levels of *clpP* and *clpX* mutants in different growth phases. In the wild-type strain AMS6, in agreement with previous findings (7, 20, 40), an increase in σ^s amount, as measured by Western analysis, began in mid- to late exponential phase and some fourfold more sigma factor was detected in the cells by the time growth ceased because of glucose exhaustion

(Fig. 1). In the *clpP* and *clpX* mutant derivatives of this strain, the early growth phase levels of σ^s were already approximately equal to the stationary-phase levels in the wild type. From the mid-exponential phase onwards, these strains also exhibited an increase in σ^s , leading to about twofold-higher levels in stationary phase (Fig. 1).

Role of chaperones. There are some striking similarities in the regulation of σ^s and the heat shock sigma factor σ^{32} (20, 23, 28, 38, 48). The levels of the latter are believed to be kept low in nonstressed cells by a complex mechanism in which several

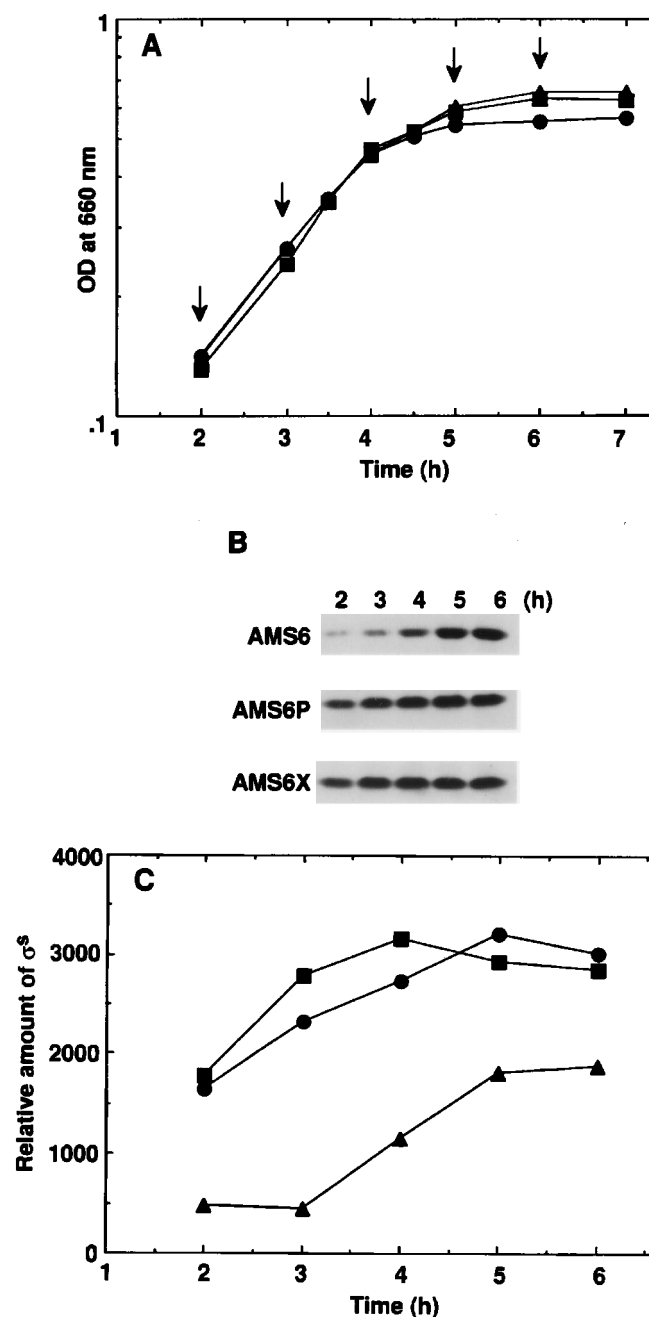


FIG. 1. (A) Growth curves of AMS6 (▲), AMS6P (●), and AMS6X (■) in 0.05% glucose-M9 medium. (B) Western blots of σ^s from cell lysates sampled at various times during the growth cycle (indicated by the arrows). (C) Densitometric quantification of the bands shown in panel B (symbols same as in panel A).

TABLE 3. Effect of *clpP* mutation on expression of single-copy transcriptional fusions of σ^s -dependent genes

Fusions	β -Galactosidase activity ^a		Ratio of β -galactosidase activity (<i>clpP</i> /wild type)
	Wild type	<i>clpP</i>	
<i>katE-lacZ</i>	147	297	2.0
<i>pexA-lacZ</i>	335	545	1.6
<i>osmY-lacZ</i>	958	2,933	3.1

^a β -Galactosidase activity was measured in Miller units in exponential-phase cells harvested at an A_{660} of 0.1 in M9 medium containing 0.03% glucose.

chaperones are postulated to have a role (38, 45, 48). To test whether chaperones have a role in the low levels of σ^s in growing cells, we measured σ^s amounts in derivatives of AMS6 defective in the following chaperones: GroEL, DnaJ, GrpE, or CbpA. None of these mutants exhibited altered levels of σ^s in exponential or stationary phase compared with the wild-type strain, as measured by Western blotting (data not shown).

Effect of *clpP* mutation on expression of σ^s -dependent transcriptional fusions. The above results show that the exponential-phase levels of σ^s in the *clpP* mutant are comparable to the stationary-phase levels in wild-type cells. Induction of several genes in *E. coli* in stationary phase depends on increased σ^s levels (22). We therefore determined if transcriptional fusions to some of these genes would exhibit higher expression in an exponential-phase *clpP* mutant. Indeed, all three transcriptional fusions that we tested showed increased expression in exponential phase in this background (Table 3): *osmY* (46) exhibited a threefold increase, and *katE* (30) and *pexA* (15) (*otsBA* [14]) each showed about a twofold increase. These increases are less than starvation-induced induction of these genes in the wild-type cells, presumably because the latter induction involves factors in addition to enhanced σ^s levels (22, 24, 40). The results show that the additional σ^s in *clpP* mutant is physiologically active.

Effect of *clpP* mutation on σ^s half-life. The above results suggest that the sensitivity of σ^s to the ClpXP protease in the exponential phase plays a role in the decreased levels of this sigma factor in this phase. To further investigate this phenomenon, we compared the half-life of σ^s in exponential- and stationary-phase AMS6 and the *clpP* mutant strain AMS6P (Table 1), using pulse-chase followed by immunoprecipitation (see Materials and Methods). In the exponential-phase AMS6 strain, σ^s had a half-life of approximately 6.5 min, but the sigma factor was virtually completely stable for the duration of the experiment in exponential-phase AMS6P (Fig. 2). In stationary-phase wild-type strain AMS6, σ^s was much more stable (half-life, >30 min) than in exponential-phase cells of this strain, confirming the previous findings (20, 39). The *clpP* mutation also made σ^s more stable in stationary phase (Fig. 2).

ClpP and ClpX levels in exponential and stationary phases. In light of the *clpP* mutation effect on σ^s half-life, a possible explanation for the increased stability of σ^s in wild-type stationary-phase cells is that the concentration of ClpP, ClpX, or both decreases in this phase. Thus, we measured the levels of these proteins during the growth cycle in cells grown in glucose-M9 medium by Western blot analysis and with ClpP and ClpX polyclonal antibodies. There appeared to be a slight increase (1.5-fold) in the levels of ClpP and ClpX during transition into the stationary phase (Fig. 3). This was supported by fusion data. An AMS6 strain containing a single-copy transcriptional *lacZ* fusion to the *clpP* gene (strain AMS1109 [Table 1]) showed a similar degree of reproducible increase as the cells entered the stationary phase.

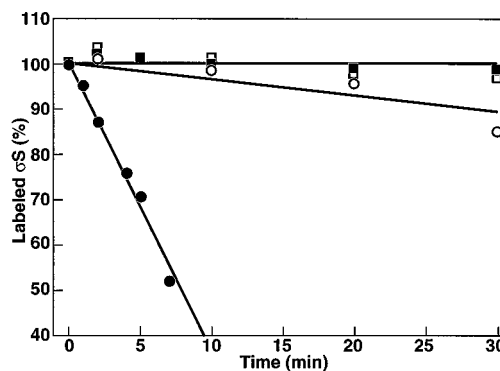


FIG. 2. Comparison of the half-life of σ^s in exponential-phase (solid symbols) and stationary-phase (open symbols) cultures in *clpP*-proficient (circles) and *clpP*-deficient (squares) backgrounds.

Recently, the DNA-binding protein H-NS (47), which is believed to have a role in DNA compaction, was shown to negatively regulate σ^s levels in exponential-phase cells (1, 44). Our Western blot analysis showed that σ^s levels in an exponential-phase *hns* mutant strain (AMS6H [Table 1]) were the same as in a *clpP* or *clpX* mutant background. Furthermore, σ^s levels in a *clpP hns* double mutant were comparable to those in a *clpP* mutant in both the exponential and stationary phases (data not shown).

Western blot analyses showed no change in the ClpXP protease concentration in the *hns* mutant throughout the growth cycle (data not shown), indicating that the H-NS effect in exponential phase was not due to its role in the synthesis or stability of this protease.

Putative σ^s target region for ClpXP protease. The σ^s protein may contain a specific region which is recognized, directly or indirectly, by ClpXP protease. To explore this possibility, we made a series of *rpoS-lacZ* translational fusions, each of which contained the complete *rpoS* promoter region (21, 39) and fragments of the coding region of this gene ranging from nt +248 to +839. Thus, strains containing these fusions should produce hybrid proteins in which 82 to 279 N-terminal amino acid residues of σ^s (i.e., 23 to 80% of the total σ^s protein) are fused to β -galactosidase. We reasoned that in a ClpXP-proficient wild-type background, these strains should exhibit different levels of β -galactosidase activity depending on whether or not they possessed the putative ClpXP target region of σ^s .

Indeed, these fusions fell into two classes in this respect.

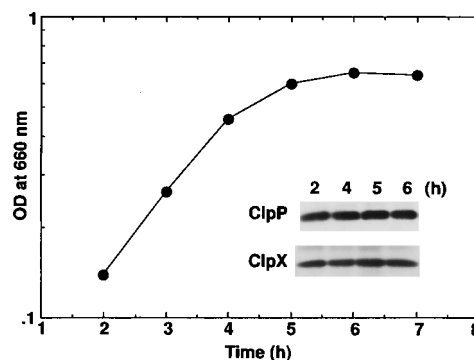


FIG. 3. Western blots of ClpP and ClpX levels in exponential- and stationary-phase *E. coli*. The growth curve of *E. coli* AMS6 in 0.05% glucose-M9 minimal medium is also shown.

TABLE 4. β -Galactosidase activity of *rpoS-lacZ* translational fusions containing different lengths of *rpoS* coding region

Class and strain	Length of <i>rpoS</i> ^a		β-Galactosidase activity ^b (Miller units)		Induction (fold)
	nt	aa	Exponential phase	Stationary phase	
Class I					
AMS154	248	82	746	1,511	2.0
AMS302	314	104	669	1,247	1.9
AMS156	479	159	707	1,506	2.1
Class II					
AMS303	563	187	380	1,332	3.5
AMS158	750	250	228	1,079	4.7
AMS304	839	279	329	1,226	3.7

^a Number of nucleotide (nt) and amino acid (aa) positions from the *rpoS* start codon, respectively.

^b Averages of more than three determinations; variations were within 15%.

Those containing 82 to 159 amino acids of the σ^s N-terminal region (Table 4, class I) showed two- to threefold-higher β -galactosidase activity in exponential phase than those with 187 to 279 amino acids of this protein (class II). Furthermore, in stationary phase, the class I fusions showed a twofold induction and class II fusions showed about a fourfold induction. This behavior is similar to the behavior of σ^s levels, as determined by Western blot analysis, in exponential- and stationary-phase wild-type (AMS6) and *clpP* mutant (AMSP) strains, respectively (Fig. 1), and suggests that class II but not class I fusions contain the σ^s region that is targeted (directly or indirectly) by ClpXP protease. If so, introduction of the *clpP* mutation should increase β -galactosidase expression in the fusion strains of the former class but not that of the latter. The data in Table 5, which provide the ratio of β -galactosidase activity of several fusions in *clpP* and *clpP*⁺ backgrounds, are consistent with this expectation. Thus, introduction of the *clpP* mutation in fusion strains with 82, 104, or 159 σ^s amino acid residues did not influence their β -galactosidase levels, but in fusions with 250 or 279 σ^s amino acid residues, this mutation increased β -galactosidase activity about fourfold. Thus, at least a part of the target region may indeed lie between amino acid residues 160 and 249 of σ^s .

The exponential-phase behavior of translational fusions containing internal deletions in σ^s protein, spanning from amino acid residues 161 to 188 (strain AMS304 Δ 1) or 173 to 188 (strain AMS304 Δ 2), was consistent with this possibility. In both strains, β -galactosidase activity in the exponential phase was about fourfold higher in the *clpP*-proficient background than in the fusion without these deletions (strain AMS304), and in a

TABLE 5. Effect of *clpP* mutation on β -galactosidase activity of *rpoS-lacZ* translational fusions containing different lengths of *rpoS* coding region^a

Strain	Fusion length (amino acids)	Ratio of β -galactosidase activity (<i>clpP</i> /wild type)
AMS154	82	0.9
AMS302	104	1.2
AMS156	159	1.0
AMS158	250	4.9
AMS304	279	3.5

^a β -Galactosidase activity was measured in exponential-phase cells grown in M9 medium containing 0.05% glucose.

TABLE 6. Exponential-phase β -galactosidase activity of *rpoS-lacZ* translational fusions containing internal deletion in σ^s protein^a

Strain	β -Galactosidase activity (Miller units)	
	ClpP ⁺	<i>clpP</i> mutant
AMS304	271	1,157
AMS304 Δ 1 ^b	964	1,003
AMS304 Δ 2 ^c	1,253	1,183

^a β -Galactosidase activities were measured in exponential-phase cells grown in M9 medium containing 0.05% glucose.

^b Deletion of 161 to 188 amino acids of σ^s protein.

^c Deletion of 173 and 188 amino acids of σ^s protein.

clpP mutant background, all three strains exhibited similar levels of activity (Table 6).

Our attempts to test the effect of these deletions on σ^s protein levels directly by Western blot analysis failed because the antibodies did not recognize the deleted sigma factor.

DISCUSSION

This investigation was undertaken to determine the physiological basis for the short half-life of σ^s in exponential-phase *E. coli* and its increased stability in stationary phase. The absence of either ClpP or ClpX from the cells made this sigma factor virtually completely stable in exponential phase, indicating that ClpXP plays a direct or indirect role in the instability of σ^s in this phase. The absence of this protease also caused a fourfold increase in the exponential-phase steady-state levels of σ^s ; thus, rapid σ^s degradation in exponential phase plays an important role in lowering the exponential-phase amount of this sigma factor.

In stationary-phase wild-type cells, σ^s exhibited higher stability and levels. Nonetheless, the absence of ClpXP protease still influenced these parameters, although less markedly: the half-life increased from >30 min to virtual total stability, and the levels increased approximately twofold. The data suggest that σ^s acquires greater resistance to the degradative activity of ClpXP as cells enter the stationary phase and that this resistance is an important factor in determining the fluctuation in σ^s levels in exponential- and stationary-phase cells.

The ClpXP protease thus performs an important role in *E. coli* physiology, since the crucial decision of whether to express growth-related genes or to differentiate into a resistant state depends on fluctuations in this sigma factor (22, 25, 26). A related finding was made by Damerau and St. John (6), who showed that *clpP* mutants failed to lower their starvation protein level when placed in a growth medium. This effect was independent of a *clpA* mutation. The existence of *clpX* was not known at that time, and the effect of its absence from the cells was not tested. In retrospect, it appears plausible that the *clpP* mutant phenotype reported by Damerau and St. John (6) was related to the inability of these mutants to lower their σ^s levels under growth conditions, as we report here.

The role in σ^s regulation is specific to ClpXP protease. Mutants deficient in other potential ATPase-bearing subunits of ClpP, i.e., ClpA or ClpB, or in Lon protease were not affected in σ^s levels in the exponential or stationary phase. The requirement that ClpX be specifically available for ClpP to affect σ^s levels and stability is consistent with the idea that the target specificity of the Clp protease system is influenced by its ATPase-bearing subunit (42, 43). For example, λ O protein is sensitive to ClpXP but not to ClpAP protease (10); in contrast, *lacZ* fusion proteins are degraded by ClpAP but not by ClpXP (41). As suggested by Gottesman and Maurizi (11), in this

respect, the ClpP system bears some resemblance to the eukaryotic proteasome, which also requires ATP for protein degradation and, depending on the associated factors, may have specificity for selected proteins (4, 11).

What accounts for the increased resistance of σ^s to ClpXP protease in the stationary phase? We show here that this is not because the ClpXP concentration decreases in stationary phase. In fact, if anything, both the Western blot and the fusion data indicated increased amounts of this protease in this phase. It remains possible, however, that despite increased amounts, the activity of this protease declines in stationary phase.

σ^s regulation resembles that of σ^{32} in some respects. In the regulation of the latter, several chaperones may play a role. It is thought that in nonstressed cells, association of σ^{32} with certain chaperones (DnaJ, DnaK, and GrpE) promotes instability. Under stressed conditions, the sigma factor dissociates from the chaperones and becomes resistant to proteolysis (38, 48). By analogy, the sensitivity of σ^s to ClpXP protease in exponential phase could arise from a similar interaction with chaperones; decomplexing from them in the stationary phase could then be the reason for its resistance to this protease. However, the lack of several chaperones (see Results) had no effect on exponential- or stationary-phase levels of σ^s , indicating that the chaperones that we tested do not have a role in this phenomenon.

It is possible that an unknown protein which inhibits σ^s degradation is synthesized in slow-growth/stationary-phase cells. Such a protein would be negatively regulated by H-NS, since σ^s is stabilized in an exponential-phase *hns* strain (1, 44; this study). Furthermore, this putative protein probably acts by enhancing, directly or indirectly, σ^s resistance to ClpXP protease. This is suggested by two results presented here: the *hns* mutation does not lower ClpXP levels, and σ^s levels in a *clpP hns* double mutant were similar to those in a *clpP* mutant background alone—had there been no interaction between the putative (H-NS repressed) protein and σ^s , one would expect higher levels of this sigma factor in the double mutant. Yamashino et al. (44) have already shown that the *hns* mutation increases σ^s levels in exponential phase, and our postulated σ^s -stabilizing protein is similar to their factor X. Our studies, however, link this protein (factor) to increased resistance of σ^s to ClpXP protease.

Studies with different-length σ^s fragments fused to LacZ protein in translational fusions suggest that amino acids 173 to 188 of the σ^s protein serve as at least a part of the target, directly or indirectly, for ClpXP protease. Fusion proteins containing fewer than 160 amino acid residues of the σ^s N-terminal region (class I fusions [Table 4]) showed the same level of expression in cells with and without ClpXP protease, but the presence of 187 or more σ^s amino acids in the fusions (class II fusions) markedly lowered expression in a ClpXP-proficient background. Results with fusions to internally deleted σ^s narrowed this putative recognition region to around amino acid residues 173 to 188 of σ^s . The β -galactosidase levels of class II fusions in different growth phases were very similar to σ^s levels in the wild type, as determined directly by Western analysis. Similarly, the levels of this enzyme in class I fusions were similar to σ^s levels in the *clpP* or *clpX* mutant (Fig. 1). This suggests that the fusion protein levels were a reliable index of fluctuations in σ^s concentration. Nonetheless, since non-recognition of truncated σ^s by antibodies precluded direct testing of these results by Western analysis, the existence of the putative recognition region remains uncertain.

Additional questions raised by the data presented here also require further studies. What accounts for the stationary-phase-induced levels of σ^s in the *clpP* and *clpX* background

(Fig. 1)? Similarly, we note that the results in Table 4 bear on the postulated mechanism of translational regulation of σ^s (23, 28). This regulation is presumed to result from the presence in the nt +101 to +187 region of the *rpoS* message of sequences complementary to the translation initiation region (20). Base-pairing between this "antisense" region and the translation initiation sequences in exponential phase masks the latter, hampering translation in this phase. If the location of the putative antisense region were indeed what has been postulated (20), a translational fusion would need no more than 210 nt of the *rpoS* coding region to show full repression in exponential phase. However, according to our results (Table 4; 28), this repression is not observed unless at least 479 nt of the coding region are present in a fusion. These aspects will be taken up in a future communication.

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ADDENDUM

After this paper was submitted for publication, Leslie Pratt sent us a preprint of a study (L. A. Pratt and T. J. Silhavy, submitted for publication) which postulates that, in regulating σ^s , ClpXP may receive environmental cues through SprE. A similar conclusion was reached by A. Muffler et al. (in press); their designation for SprE is Rssb.

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